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14. ABSTRACT

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions; host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis we will examine the disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. This system will provide details into the host factor(s) involved in transport of prions to cells in the LRS. We have shown the susceptibility of HY and DY TME to phagocytosis and degradation by a murine macrophage cell line. We are currently studying the effects of prion infection on phagocytic ability and cell viability. We have shown differences in the spatial and temporal spread of the HY and DY TME agents in LRS tissues following intraperitoneal inoculation. We have shown gender specific responses to intraperitoneal DY TME inoculation.

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Prion diseases, macrophage, complement

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Introduction

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions, host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis two animal models will be used. Genetically engineered mice that lack components of the complement system will be used to test the hypothesis that complement binding to PrP^{Sc} is involved in targeting of prions to cells in the spleen and uptake by macrophages. A second system will examine disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to test the hypothesis that DY TME is not bound by complement resulting in its absence in the spleen. The mouse and hamster systems investigate prion interactions with complement components based on differences of host and strain properties, respectively. This study will provide details into the host factor(s) involved in transport of prions to cells in the LRS, such as spleen.

Body

Tissue distribution of HY & DY TME at early time points post-infection. As previously reported, we began to investigate the distribution of infectivity and PrP^{Sc} in hamsters infected with the HY and DY TME agent at early time points post-infection as outlined in task 4. In the previous experiment, hamsters were intraperitoneally inoculated with $10^{5.5}$ LD_{50} of DY TME or $10^{8.5}$ LD_{50} of HY TME. At 1, 2, 4, 8, 16, and 32 hours post-infection, three animals for each inoculation group were sacrificed and peritoneal cells, spleen, mesenteric lymph node, medial iliac lymph node, and submandibular lymph node were collected. We used detergent extraction and ultracentrifugation to enrich for PrP^{Sc} prior to Western blot analysis. Our results indicated differences between the two TME strains, and we repeated the experiment expanding the time points to include 64 hours, 1, 2, 4, 6, 8, and 11 weeks post-infection (Tables 1 and 2).

Table 1. Distribution of PrP^{Sc} following intraperitoneal inoculation with the HY TME agent

	1h	2h	4h	8h	16h	32h	64h	1wk	2wk	4wk	6wk	8wk	11wk
Peritoneal Cells	9/9[a]	7/9	8/9	6/6	5/6	5/6	4/6	3/6	1/6	0/6	0/3	0/3	0/3
Medial Iliac LN	5/12	5/9	4/12	2/6	3/6	3/6	0/6	0/6	0/6	0/6	0/3	0/3	0/3
Mesenteric LN	0/9	2/9	2/12	1/6	1/6	1/6	0/6	0/6	0/6	0/6	0/3	0/3	0/3
Spleen	3/12	5/12	8/12	2/6	0/6	1/6	0/6	0/6	0/6	0/6	0/3	2/3	0/3

[a] number of PrP^{Sc} positive animals/number of inoculated animals

Table 2. Distribution of PrP^{Sc} following intraperitoneal inoculation with the DY TME agent

	1h	2h	4h	8h	16h	32h	64h	1wk	2wk	4wk	6wk	8wk	11wk
Peritoneal Cells	8/9[a]	9/9	9/9	5/6	2/6	2/6	0/3	0/3	0/3	0/3	ND[b]	ND	ND
Medial Iliac LN	0/12	3/9	2/12	1/6	1/6	2/6	0/3	0/3	0/3	0/3	ND	ND	ND
Mesenteric LN	0/12	0/9	0/12	0/6	0/6	0/6	0/3	0/3	0/3	0/3	ND	ND	ND
Spleen	4/12	4/12	4/12	0/6	0/6	0/6	0/3	0/3	0/3	0/3	ND	ND	ND

[a] number of PrP^{Sc} positive animals/number of inoculated animals
[b] not done

HY PrP^{Sc} is present in the peritoneal cell collections from 1 hour post-infection through 2 weeks post-infection. HY PrP^{Sc} is present in the medial iliac lymph node and spleen from 1 hour post-infection to 32 hours post-infection. HY PrP^{Sc} is present in the mesenteric lymph node by 2 hours post-infection, and remains in this tissue through 32 hours post-infection (Table 1). In contrast, DY PrP^{Sc} is present in the peritoneal cells from 1 hour post-infection through 32 hours post-infection. DY PrP^{Sc} is detectable in the medial iliac lymph node from 2 hours post-infection to 32 hours post-infection. DY PrP^{Sc} is undetectable in the mesenteric lymph node at all time points, and is detectable in the spleen between 1 and 4 hours post-infection (Table 2).

These data indicate that both HY and DY TME agents are transported to secondary lymphoreticular system tissues following intraperitoneal inoculation. The inability of DY TME to cause disease following intraperitoneal inoculation does not appear to be related to a lack of transport of the agent from the periphery to the draining lymph nodes. DY TME is transported to the medial iliac lymph node, the local, draining lymph node of the peritoneal cavity, in a time frame similar to HY TME.

To confirm our results, we will analyze secondary lymphoreticular system tissues using NaPTA precipitation. In this study, hamsters will be intraperitoneally inoculated with uninfected homogenate or $10^{5.5}$ LD_{50} of DY TME or $10^{8.5}$ LD_{50} of HY TME. At 1, 2, 4, 8, 16, 32, 64 hours post-infection, and 1, 2, 4, 6, 8, 10, 12 and 14 weeks post-infection three animals for each inoculation group will be sacrificed and the peritoneal cells, medial iliac lymph node, mesenteric lymph node, submandibular lymph node, and spleen will be collected. Following tissue homogenization, the PrP^{Sc} content of the spleen, medial iliac lymph node, mesenteric lymph node, and peritoneal cells will analyzed following NaPTA precipitation and Western blot analysis using the prion protein specific antibody, 3F4, as previously described (Wadsworth et al., 2001).

Macrophage degradation of TME PrP^{Sc}. The interactions of HY and DY TME PrP^{Sc} and macrophages are being investigated as outlined in task 6. Significant progress has been made co-culturing HY and DY TME with a murine macrophage cell line (RAW 264.7 cells, ATCC, Manassas, VA). In these experiments, RAW 264.7 cells were grown to confluence in DMEM (ATCC, Manassas, VA) containing 10% fetal bovine serum (ATCC, Manassas, VA) and 100 U/ml of penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO). The cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA) and the cell concentration was adjusted to 10^6 cells per ml using pre-warmed

(37°C) DMEM Media. A total of 10^5 cells per well were placed in a plastic 96-well cell culture plate. The cells were allowed to adhere to the plate for 1 hour.

To assess if RAW 264.7 cells could degrade HY or DY PrP^{Sc}, PrP^{Sc} was incubated with either 10^5 RAW 264.7 cells or an equal volume of DMEM Media without cells as a control for non-macrophage mediated PrP^{Sc} degradation. HY and DY TME brain homogenates were prepared in PBS and were not digested with proteinase K. 250 µg equivalents of brain homogenate in DMEM Media were added to each well. Samples in triplicate were collected at 1, 2, 4, 6, 12, 18, 24, 48, and 72 hours post-TME-infection. At each collection point the media was removed and saved. An equal volume of DMEM Media was added to the well to collect the remaining free PrP^{Sc} and was added to the previously collected media. The PrP^{Sc} in the media and wash is referred to as the media associated PrP. To the cells, 100µl of 0.1% w/v NLS was added to the well to dislodge the cells from the well and collected. The wells were then washed with 100 µl of 0.1% w/v NLS that was added to the first cell collection. The PrP^{Sc} collected in 0.1% NLS is referred to as cell-associated PrP. The levels of PrP in the media and associated with the cells were quantified using Western blot analysis with the anti-PrP monoclonal antibody, 3F4.

Using this system, we have shown the ability of murine macrophage (RAW 264.7 cells) to degrade PrP^{Sc}. In HY TME-infected macrophages there was a significant reduction in PrP^{Sc} levels in the media through 72 hours post-infection (Figure 1, Panel A). In macrophages infected with HY TME brain homogenate, the cell associated PrP^{Sc} level was detectable at low levels at 1 hour post-infection, the PrP^{Sc} levels increased through 24 hours post-infection, and was still detectable at 72 hours post-infection (Figure 1, Panel A). In DY TME infected macrophages there was also a significant reduction in the levels of PrP^{Sc} in the media by 72 hours post-infection (Figure 1, Panel B). In macrophages infected with DY TME-infected

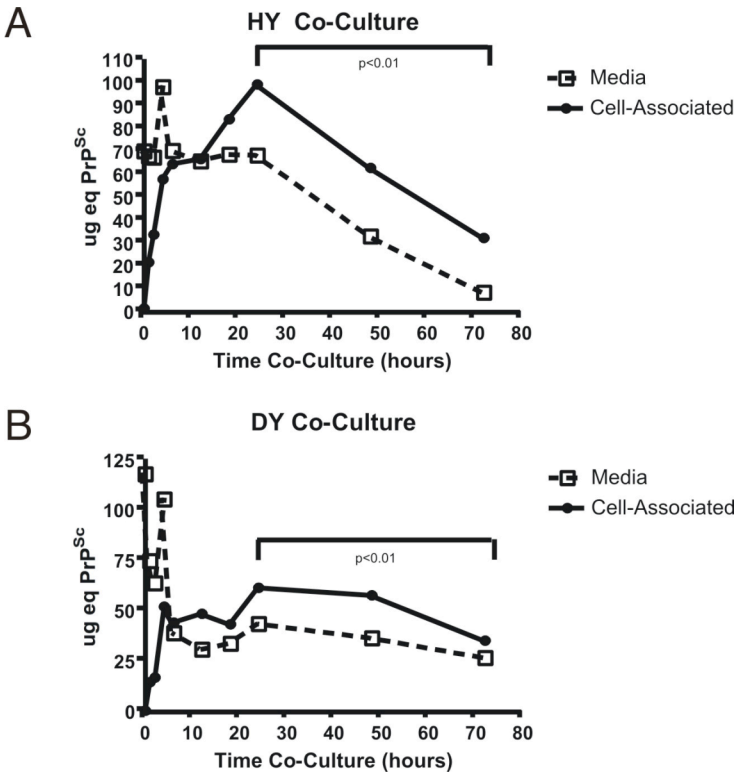

Figure 1. Abundance of HY or DY PrP^{Sc} (ug eq PrP) in the media or associated with murine macrophage RAW 264.7 cells at selected time points (1, 2, 4, 6, 12, 18, 24, 48, & 72 hours) post-infection.

homogenate, cell-associated PrP^{Sc} was first detected at 4 hour post-infection and PrP^{Sc} levels increased until 24 hours when levels were significantly reduced by 72 hours post-infection (Figure 1, Panel B).

To summarize, the murine macrophage cell line RAW 264.7 cells degrade both HY and DY PrP^{Sc}. Cell-associated PrP^{Sc} levels peaked by 24 hours, and were significantly degraded by 72 hours. This suggests that both HY and DY TME may be phagocytosed and degraded by lymphatic tissue macrophages. Although our initial experiments with primary hamster adherent peritoneal cells indicated a differential degradation of HY and DY TME, these data suggest that the two strains are processed by murine macrophages in a similar manner. The inability of DY TME to cause disease following intraperitoneal inoculation does not appear to be relater to faster uptake and/or degradation of the agent.

To further our studies, we are currently examining the effect of PrP^{Sc} on the macrophage phagocytic ability and cell viability using commercially available kits. We will be using the phagocytosis blocker, cytochalasin D to examine the media and cell-associated PrP^{Sc} content when phagocytosis is blocked prior to co-culture with the TME agents.

Serum amyloid protein and the response of female hamsters to DY TME-infection. It is possible that the observed increase in incubation period in complement deficient animals is not due to a direct interaction of complement components with PrP^{Sc} but via an intermediate molecule. A possible candidate molecule is serum amyloid protein (SAP) that has been shown to bind amyloid and can also directly bind to C1q (Coe and Ross, 1990; Nauta et al., 2003). The hamster homolog of SAP is female protein, which is regulated by estrogen resulting in approximately 100-fold higher serum levels of female protein compared to male hamsters (Coe & Ross, 1990).

According to previously published data, intraperitoneal inoculation of male hamsters with the DY TME agent derived from males did not result in the animals expressing clinical symptoms and the DY TME agent was undetectable in secondary lymphoreticular tissues and brain (Bartz et al., 2005). To investigate if higher levels of serum protein had an effect on the ability of DY TME to cause disease following intraperitoneal inoculation, additional experimentation was necessary. DY TME derived from male and female hamsters were intraperitoneally inoculated into male and female recipient hamsters (Table 3).

Table 3. Intraperitoneal inoculation of male and female hamsters with DY TME

Gender	Inoculum	# positive/# inoculated	Incubation Period
Male	Male	1/6[a]	341 dpi[b]
Female	Male	1/6	399 dpi
Male	Female	4/6	399, 264, 264, 336 dpi
Female	Female	1/6	287 dpi

[a] number of PrP^{Sc} positive animals/number of inoculated animals
[b] days post-infection

The hamsters were assessed daily for the onset of clinical symptoms of DY TME which is characterized by a progressive lethargy. At the time of this report, at least one of the animals from each inoculation group has shown DY TME clinical

symptoms (Table 3). One out of the six male hamsters inoculated with male DY TME exhibited clinical symptoms at 341 days post-infection. One out of the six female hamsters inoculated with male DY TME exhibited clinical symptoms at 399 days post-infection. Four out of the six male hamsters inoculated with female DY TME exhibited clinical symptoms at 264, 264, 336, and 399 days post-infection. One out of the six female hamsters that was inoculated with female DY TME exhibited clinical symptoms at 287 days post-infection (Table 3). DY PrP^{Sc} was present in the brains of each of these animals at the time of sacrifice (Figure 2, lanes 2, 4, 6, 8, 10, 12, and 14, respectively), which can be shown by the migration of the unglycosylated band at 19kDa. This is the first

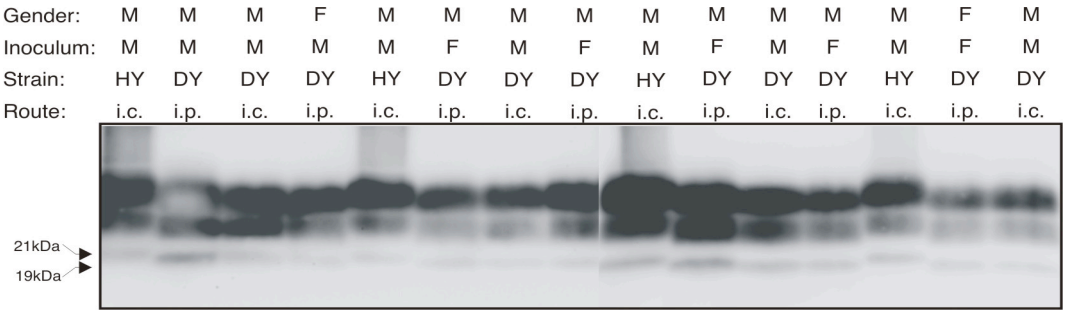

Figure 2. Western blot analysis of brain homogenate from male and female hamsters inoculated with DY TME. (M-male, F-female, i.c.-intracerebral, i.p.-intraperitoneal). The 19kDa migration of the unglycosylated band from the experimental groups (intraperitoneal inoculation) is compared with the migration of the unglycosylated band from the control groups (intracerebral inoculation). HY TME migrates at 21kDa, DY TME migrates at 19kDa.

known case of DY TME causing disease following a non-neuronal route of inoculation. To further study the role of SAP in peripheral DY TME pathogenesis, we will repeat this experiment and collect secondary lymphoreticular tissues at early time points post-infection including 1, 2, 4, 8, 16, 32, 64, and 128 hours post-infection as described in section 1. We will also examine the biochemical properties of the DY PrP^{Sc} derived from these animals by proteinase K digestion and Guanadium denaturation.

Key Research Accomplishments

1. Peritoneal cells and lymphoreticular system tissues from uninfected, HY TME and DY TME-infected hamsters have been collected through 11 weeks post-infection and the spatial and temporal spread of PrP^{Sc} in these tissues has been determined by Western blot analysis.
2. Peritoneal cells and secondary lymphoreticular system tissues from HY TME and DY TME-infected hamsters are being collected and we are currently using NaPTA precipitation and Western blot analysis to verify the spatial and temporal spread of PrP^{Sc}.
3. Co-culture experiments of murine macrophage RAW 264.7 cell line and PrP^{Sc} from HY and DY TME-infected hamsters demonstrated PrP^{Sc} phagocytosis and degradation.

4. Effects of prion infection on macrophages are being determined via cell viability and phagocytosis assays.
5. Gender specific specificity to DY TME intraperitoneal infection has been determined.

Reportable Outcomes

None

Conclusions

We have initiated studies on the tissue distribution of HY and DY TME at early time points post-intraperitoneal infection. We have collected peritoneal cells and lymphoreticular system tissues and have of analyzed these tissues for the presence of PrP^{Sc} through 11 weeks post-infection. We are currently analyzing tissue that was collected in a subsequent experiment to verify the presence of PrP^{Sc} using NaPTA precipitation and Western blot analysis. We have shown phagocytosis and degradation of PrP^{Sc} by the murine macrophage cell line RAW 264.7 cells. We have begun to investigate the effects of prion infection in macrophages using cell viability and phagocytosis assays. Finally, we have demonstrated that both male and female hamsters are susceptible to DY TME intraperitoneal infection.

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Appendices

None